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Progress on determining the function of *BRCA1* suggests its involvement in DNA repair and transcriptional regulation. BRCA1 C-terminus acts as a transactivation domain and introduction of germ-line mutations, but not benign polymorphisms, abolishes transcriptional activation, suggesting a critical role for this function of BRCA1 in cancer development. Our hypothesis is that BRCA1 transactivation function is regulated by an intramolecular interaction. During the past year we focused on two specific aims: *1) map the interaction sites; 2) define the in vivo dominant negative activity of truncations in BRCA1 that retain the inhibitory domain but disrupt the transactivation domain.* We optimized transfection conditions and selected proper expression vectors for our analysis. In addition, we obtained several constructs containing BRCA1 fragments and confirmed protein expression in mammalian and yeast cells that will serve as important tools. A yeast two-hybrid approach and an interaction assay in mammalian cells were used to identify the interaction sites. However, while results from our interaction assay in mammalian cells are promising, the yeast two-hybrid system is not adequate due to the high background presented by the BRCA1 fragments. We have also investigated a yeast-based assay to screen for the dominant negative activity of various BRCA1 alleles.

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FOREWORD

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INTRODUCTION

Recent evidence points to the involvement of BRCA1 in two basic cellular processes: DNA repair and transcriptional regulation. BRCA1 is present in a complex containing Rad51 (1) and BRCA2 (2) and DNA damage may control BRCA1 phosphorylation and subnuclear location (3,4), strongly suggesting its involvement in the maintenance of genome integrity. Additional evidence for the role of BRCA1 in maintenance of genome integrity is provided by targeted disruption of *Brca1* in the mouse. Mouse embryos lacking *Brca1* are hypersensitive to γ-irradiation and cells display numerical and structural chromosomal aberrations (5).

We and others have shown that BRCA1 C-terminus has the ability to activate transcription in mammalian and yeast cells and that the introduction of germ-line disease-associated mutations, but not benign polymorphisms, abolishes this activity (6-8). BRCA1 can be copurified with the RNA polymerase II holoenzyme supporting the idea that BRCA1 is involved in transcription regulation (9,10). In addition, BRCA1 causes cell-cycle arrest via transactivation of $p21^{WAF1/CiP1}$ (11) and regulates p53-dependent gene expression, acting as a coactivator for p53 (12,13). In all of these studies, the C-terminal region was necessary for activity. It is still not clear whether BRCA1 is a multifunctional protein with repair and transcription regulation functions or whether the role of BRCA1 in repair is mediated through transcription activation. In either case, these functions are not necessarily mutually exclusive.

In the original proposal we presented preliminary evidence identifying a critical region for the modulation of transcriptional activation by BRCA1. Our results suggested the existence of an intramolecular interaction domain that masks the activation domain of BRCA1, thus impairing the recruitment of the transcription initiation complex. Our working hypothesis is that BRCA1 transcriptional activation function is regulated by an autoinhibitory intramolecular interaction between regions encoded by exon 12 and the C-terminal region comprising the BRCT domains. This mechanism may form the basis of the regulation of transcriptional activation by BRCA1. We expect that a region that interacts and masks the activation domain will be identified in detail and will reveal one of the means by which BRCA1 activity is regulated.

BODY

During the past year we focused on the two specific aims described in our proposal and in the work statement:

Task 1. **To map the sites involved in the intramolecular interaction in BRCA1 using the yeast two-hybrid assay (months 1-12).**

- Develop a series of plasmids for expressing the BRCA1 region aa 1366-1455 and smaller fragments as in-frame fusions with the GAL4 DNA binding domain and activation domain.
- Develop a series of plasmids for expressing the BRCA1 minimal transactivation domain and smaller fragments as in-frame fusions with the GAL4 DNA binding domain (with the exception of fragments that activate transcription alone) and activation domain.
- Perform yeast two-hybrid assays using the interaction region and the MTD to confirm the interaction and pinpoint the exact location.
- If a problem arises, such as the impossibility of testing a particular construct because of non-specific activation we will subclone the constructs in GST-fusion and FLAG-tagged vectors.
- Express the tagged fragments in mammalian cells and check the interaction by immunoprecipitation and western blotting (e.g. IP with FLAG antibody and blot against GST antibody).

Task 2. **To define the in vivo inhibition and the dominant negative activity of truncations of BRCA1 (months 8-24).**

- Develop a series of plasmids for expressing the BRCA1 region aa 1366-1455 and smaller fragments (as defined in Task 1) in mammalian cells.
- Optimize transient transfections of breast cancer cell lines and in ovarian cancer cell lines.
- Confirm that expression of the interaction domain blocks the activity of BRCA1 in inducing a reporter luciferase gene.

KEY RESEARCH ACCOMPLISHMENTS

Progress on goals defined in Task 1.

- In an attempt to identify the interaction region we have developed a series of plasmids for the expression in yeast of BRCA1 fragments as in-frame fusions with the GAL4 DNA binding domain and activation domain. The following constructs were made as fusions to GAL4 DNA binding domain (DBD) in the pGBT9 vector backbone (Clontech): BRCA1 (aa 1366-1803); (aa 1396-1778); (aa 1366-1778); (aa 1366-1803); (aa 1366-1718); (aa 1366-1559); (aa 1-302); (aa 302-1313); (aa 1560-1803); (aa 1366-1455). The following constructs were made as fusions to GAL4 Activation domain (AD) in the pGAD424 vector backbone (Clontech): BRCA1 (aa 1366-1778); (aa 1560-1863); (aa 1-1863).

In order to start narrowing down the binding site, we transformed these constructs in yeast to confirm our initial results. The yeast two-hybrid assays performed with these constructs confirmed that a region comprising exon 12 was indeed interacting with regions in the C-terminal domain of BRCA1. However, the results were confounded by the high background found in many fragments of BRCA1. Since many constructs displayed this activity it became difficult to design experiments to avoid these confounding effects. We also found that protein expression levels varied significantly in the clones studied and generated high variability and low reproducibility of the results in assays performed in different days. We concluded that the mapping of the interaction site couldn't be done unequivocally using the yeast-based approach. We are now preparing mammalian expression vectors expressing tagged versions of the fragments to perform co-immunoprecipitation studies. We have made the following constructs on a pCMV-FLAG (Kodak) vectors: BRCA1 aa (1366-1778); (aa 1366-1455); (aa 1366-1559) and (aa 1366-1718). We have confirmed expression of the constructs in 293T cells. We are now in the process of performing the co-immunoprecipitation assays.

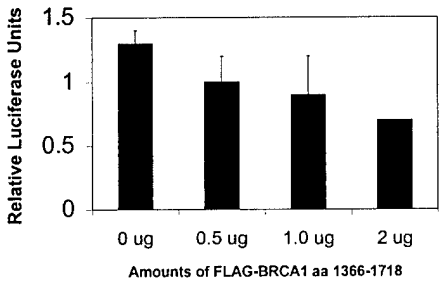
Progress on goals defined in Task 2.

- Our initial step was to optimize the assay to define which fragment of BRCA1 was able to inhibit transcription by GAL4 DBD aa 1560-1863 or aa 1396-1863 when expressed in *trans*.

In a series of transfections, we tested cell lines (293T and COS-7), transfection reagents (Lipofectamine, Fugene 6 and Calcium phosphate) and internal transfection control (pRL-TK and pRL-SV40). We found that 293T cells display the highest transfection efficiency and no signs of toxicity when transfected with Fugene 6. Our internal control is a *Renilla* luciferase under the control of a constitutive promoter (we tested SV40 and TK promoter). pRL-SV40 was shown to be affected by BRCA1 expression whereas pRL-TK was not and was thus chosen as an appropriate control. We also tested a pCDNA3-based expression (instead of pSG424) GAL4 DBD (aa 1396-1863) but found its expression levels to be too high and not susceptible to inhibition by ectopic expression by any other BRCA1 construct. We used the FLAG-tagged constructs described above to perform the experiments. Using the above mentioned conditions we performed a preliminary experiment. As shown in Figure 1, a FLAG-tagged BRCA1 fragment (aa 1366-1718) was able to inhibit transcription mediated by the GAL4 DBD BRCA1 (aa 1560-1863). Similar to the experiment reported in the proposal (using aa

1366-1778), this initial experiment supports our notion of an interaction domain in BRCA1. We are now in the process of repeating these experiments and performing different controls to start to narrow down the interaction site (next step will be to test aa 1366-1559 and a control using GAL4 DBD p53 to test whether these effects are specific for BRCA1).

Figure 1. Typical experiment showing the effects of expression of FLAG-BRCA1 aa 1366-1718 on transcription mediated by GAL4 DBD: BRCA1 aa 1560-1863.



ADDITIONAL ACHIEVEMENTS

In parallel to the experiments described above, we investigated the possibility that a yeast assay based on the small colony phenotype assay (15) could be used to detect in vivo dominant activity function of certain BRCA1 alleles. For that we developed a series of plasmids carrying full-length wild type and mutant BRCA1 in plasmids with both *LEU2* and *TRP1* selective markers (see reportable outcome). Our hypothesis was that if a mutant BRCA1 displayed a dominant-negative activity it would rescue the small colony phenotype and yeast colonies would be large. We performed the tests in four different yeast strains (TGY14, EGY48, SFY526 and HF7c). However, we found that in all strains tested there were an unacceptable high number of large colonies (probably revertants) that hampered analysis. We then investigated the possibility that the variable effects were due to the BRCA1 plasmids being maintained episomally at high copy number. We decided to integrate a copy of BRCA1 in yeast under an inducible promoter. Although we were able to generate a yeast cell strain carrying an inducible BRCA1, expression levels were not sufficient to promote the small colony phenotype needed to perform the assay.

In order to attempt identification of proteins that interact with the region comprising exon 12 and 13, we decided to perform a yeast two-hybrid screen using pGBT9 (aa 1366-1455) and screening against a mammary gland cDNA library. The screen was performed using 50 mM 3-Aminitriazol to reduce the background displayed by the bait plasmid. Over 300 clones were isolated after growth in selective medium. Of these, 140 were shown to be double positive (also activated endogenous β-galactosidase reporter. We are now in the process of isolating the DNA from yeast colonies and retransforming to check for interaction. At this point we have identified 6 independent cDNAs that are positive upon retransformation. These cDNAs are now being sequenced and will be subcloned into mammalian two-hybrid vectors to confirm interaction in mammalian cells. We also performed a screen using pGBT9 (aa 1366-1778) but interestingly, we did not isolate any double positive clones.

One additional way to evaluate the functional importance of a particular region of a protein is to analyze its phylogenetic conservation. However, the high level of sequence divergence in BRCA1 makes it difficult to rely on results from computer analysis. For example, large proteins that contain a RING finger and BRCT domains may look like a BRCA1 homolog but be completely evolutionarily unrelated. Therefore, we started a collaboration with laboratories in Australia (Dr. Scott Andrew, Kolling Inst., North Shore Hospital – Sydney) and in South America (Dr. Turan Urmenyi, Federal University of Rio de Janeiro; Dr. Sandro Souza, Ludwig Institute, São Paulo) to clone BRCA1 from primitive (marsupials and monotremes) mammals. We have identified sequences in the South American possum homologous to human BRCA1 and we will use them as homologous probes to clone

BRCA1 from a cDNA library from possum's testes. Dr. Elaine Ostrander's group has embarked on a similar project and we are communicating our results to avoid duplication.

We were invited to write a review on the subcellular localization of BRCA1 that appeared in the January issue of Histology and Histolpathology (copy attached). Also, in a different project (not funded by this grant we performed a extensive mutagenesis analysis of the C-terminal region of BRCA1 that will be instrumental in analyzing our future results on transcriptional activation. This paper, the first paper of primary data from the laboratory was published in the May 1 issue of Cancer Research (copy attached).

REPORTABLE OUTCOMES

- A yeast strain with an inducible expression of human BRCA1.
  TGYBRU. Yeast strain made on a TGY14 background expressing an integrated single copy wild type full length BRCA1 under the control of an inducible (GAL1) promoter.

- An invited review on the function of BRCA1 (14; copy attached).

- A series of yeast expression plasmids.

GAL DBD and AD fusions:

pGBT9 BRCA1 (aa 1366-1803)	pGBT9 BRCA1 (aa 1396-1778)
pGBT9 BRCA1 (aa 1366-1778)	pGBT9 BRCA1 (aa 1366-1803)
pGBT9 BRCA1 (aa 1366-1718)	pGBT9 BRCA1 (aa 1366-1559)
pGBT9 BRCA1 (aa 1-302)	pGBT9 BRCA1 (aa 302-1313)
pGBT9 BRCA1 (aa 1560-1803)	pGBT9 BRCA1 (aa 1366-1455)
pGAD BRCA1 (aa 1366-1778)	pGAD BRCA1 (aa 1560-1863)
pGAD BRCA1 (aa 1-1863)	pACT2 BRCA1 (aa 1-1863)

No fusions:

p425GPD BRCA1 1853X	p425GPD BRCA1	p425GPD BRCA1 C61G
p425ADH BRCA1	p424GPD BRCA1	pJG4-4 BRCA1

Integrating vectors:

BB25 BRCA1	BB301 BRCA1

- A series of mammalian expression FLAG-tagged plasmids.

FLAG-tagged vectors:

pCMV-FLAG (aa 1366-1778)	pCMV-FLAG (aa 1366-1455)
pCMV-FLAG (aa 1366-1559)	pCMV-FLAG (aa 1366-1718)

GAL4 DBD fusions:

pSG424 (aa 1396-1863)	pCDNA3 SG (aa 1560-1863)
pCDNA3 SG (aa 1396-1863)	

No fusions:

pCDNA3 (aa 1366-1778)	pCDNA3 (aa 1560-1863)
pCDNA3 1853X	

CONCLUSIONS

In the first year of our project we were able to achieve most of the goals defined in our original proposal. Importantly, we generated a series of expression plasmids that will be instrumental in future experiments. The inability to use the yeast two-hybrid approach to advance in our fine mapping of the interaction domain was a setback, but we had anticipated possible problems of that nature and were able to provide an alternative approach. We also used this initial phase of the project to optimize conditions and reagents to perform future experiments. This usually is a time-consuming phase of the studies but we believe it is important to guarantee the best conditions for our experiments as well as confirming expression of the different fragments in mammalian cells. With the optimized conditions and reagents we should be able to quickly identify the interaction domains relevant for regulation of transcriptional activity. In addition, we have explored several new avenues relating to the activity of BRCA1 (see Additional Achievements). In summary we believe this initial phase of the project was highly positive despite the technical problems encountered.

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APPENDIX

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Histology and Histopathology

Cellular and Molecular Biology

Invited Review

A nuclear function for the tumor suppressor BRCA1

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Summary. The breast and ovarian cancer susceptibility gene *BRCA1* has been recently cloned and revealed an open reading frame of 1863 amino acids, but a lack of significant homology to any known protein in the database has led to few clues about its functions. One of the first steps to investigate the function of BRCA1 was to define its subcellular localization. Several reports have led to contradictory findings that include: nuclear localization in normal cells and cytoplasmic in breast and ovarian cancer cells; nuclear in both normal and cancer cells; cytoplasmic and secreted to the extracellular space; present in tube-like invaginations of the nucleus; and colocalizing with the centrosome. As is apparent, the subcellular localization has been the most controversial aspect of BRCA1 biology and is a key point to uncover its functions. In this paper we review the published data on subcellular localization of BRCA1 with special emphasis on the antibodies and techniques used. We conclude that there is now overwhelming evidence to support a nuclear localization for BRCA1, both in normal and cancer cells. In addition, several BRCA1-interacting proteins have been isolated and they are preferentially located in the nucleus. Evidence supporting a physiological function for BRCA1 during DNA repair and transcriptional activation is also discussed.

Key words: Breast cancer, Tumor suppressor gene, BRCA1, Antibody specificity

Abbreviations: GST: Glutathione-S-transferase; NLS: nuclear localization signal; CEFs: chicken embryo fibroblasts; SDS: sodium dodecyl sulfate; DAPI: 4',6-diamidino-2-phenylindole; FBS: fetal bovine serum; PBS: phosphate buffered saline

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Introduction

Mutations in *BRCA1* account for approximately 45% of the families with high incidence of breast cancer and 80% of families with high incidence of both breast and ovarian cancer (Easton et al., 1993). Identification of human *BRCA1* by positional cloning techniques revealed an open reading frame coding for 1863 amino acids with no statistically significant homology to proteins in the database, with the exception of a zinc-binding RING finger motif (C3HC4) in the N-terminal region (Miki et al., 1994). This motif is found in several proteins that have their functions mediated through DNA binding (Saurin et al., 1996). In addition, the presence of two putative nuclear localization signals (NLSs; aa 500-508 and 609-615) and an excess of negatively charged residues in the C-terminal region of BRCA1 suggested a function for *BRCA1* in transcriptional regulation (Miki et al., 1994). Using a different computational strategy, Koonin et al. partitioned the BRCA1 sequence into putative globular and non-globular domains and used the globular domains to perform iterative searches in the database (Koonin et al., 1996). These studies defined a globular domain repeated in tandem in the C-terminal region of BRCA1, named BRCT (for BRCA1 C-terminal domain) also present in 53BP1, a p53 binding protein (Koonin et al., 1996). This study was later extended and defined a superfamily of proteins containing the BRCT domains involved in DNA damage and cell cycle checkpoints (Bork et al., 1997; Callebaut and Mornon, 1997). Collectively, the fact that the RING finger, the NLSs, the excess of negatively charged residues and the BRCT domains are conserved in human, dog, rat and mouse *Brca1*, suggests that these regions are significant for BRCA1 function (Abel et al., 1995; Lane et al., 1995; Sharan et al., 1995; Szabo et al., 1996; Bennet et al., 1999).

Is BRCA1 aberrantly localized in breast and ovarian cancer?

One of the first strategies to understand the biochemical function of BRCA1 was immunofluorescence

and immunocytochemical analysis. Chen et al. (1995a,b) initially characterized BRCA1 as a 220 kDa nuclear phosphoprotein in normal cells as well as in cells derived from tumors other than breast and ovarian cancer. Interestingly, in the majority of breast and ovarian cancer cell lines and cells obtained from malignant pleural effusions of these tumors, BRCA1 appeared to be mislocalized to the cytoplasm (Chen et al., 1995a,b). Previous experiments raised questions about the role of BRCA1 in sporadic breast and ovarian cancer since no mutations in BRCA1 had been found in sporadic breast cancers and very few in ovarian cancers (Futreal et al., 1994). Mislocalization of BRCA1 to the cytoplasm suggested that a deficient nuclear transport mechanism might disrupt BRCA1 function in sporadic tumors in the absence of loss-of-function mutations. However, these authors only performed biochemical fractionation in HBL100 cells, and there was no fractionation data of the cancer cell lines showing mislocalization (Chen et al., 1995a,b).

In 1996, Scully et al. (1996a) reexamined the question by using an affinity purified polyclonal antibody as well as seven monoclonals (for a comprehensive list of BRCA1 antibodies published in the literature see Table 1; for a comparative study of several antibodies see Wilson et al., 1999) raised against various epitopes and found a consistent "nuclear dot" pattern in cell lines fixed with neutral paraformaldehyde, or methanol, or 70% ethanol. Moreover, biochemical fractionation analysis of three cancer cell lines (SKOV-3, MCF-7 and U20S) confirmed the presence of BRCA1 in the nuclear but not in the cytoplasmic fractions (Scully et al., 1996a). Although some of the antibodies showed weak cytoplasmic staining, confocal microscopy studies could not demonstrate colocalization of the signals derived from different antibodies, strongly arguing for non-specific cross-reactivity in the cytoplasm. Paraffin-embedded sections fixed with alcoholic formalin were shown to generate nuclear, both nuclear and cytoplasmic, as well as cytoplasmic staining. However, when these sections were treated with microwave heating, the staining was predominantly cytoplasmic suggesting that artifacts due to sample preparation may contribute to the confusion in BRCA1 location. In fact, cell lines where BRCA1 had been shown to be nuclear both by subcellular fractionation of unfixed cells and by immunostaining, displayed variable results when subjected to different fixation and heating conditions (Scully et al., 1996a).

To circumvent specificity problems with antisera to native BRCA1 epitopes, Chen et al. (1995b) ectopically overexpressed an N-terminal FLAG-tagged BRCA1 to show the tagged protein to be in the nucleus of normal cells but in the cytoplasm in a series of breast cancer cell lines. A caveat of ectopic overexpression is that high levels of protein can saturate subcellular compartments and result in the presence of the protein where it is not normally found under physiological conditions. Moreover, overexpression of BRCA1 can also cause toxicity and induce changes in cell morphology (Wilson et al., 1997). An important issue that remains is the localization of BRCA1 in rapidly proliferating cells versus contact-inhibited cells. Contact-inhibited cells have very low, in many cases undetectable, BRCA1 levels (Chen et al., 1996a; Jin et al., 1997). This may also be a source of artifactual results because antibodies that show both nuclear and some cytoplasmic staining will show only cytoplasmic staining when cells are contact inhibited due to the absence BRCA1 and presence of cross-reacting species in the cytoplasm. Examining the published results, cells presenting cytoplasmic staining are found in close contact with other neighboring cells. Immunostaining of cells expressing BRCA1 (HBL100) and chicken embryo fibroblasts (CEFs) that lack BRCA1 judged by low stringency southern blots (Miki et al., 1994), is a particularly revealing example of cytoplasmic cross-reactivity (Fig. 1A). Cells expressing BRCA1 (HBL100), show both a nuclear dot pattern and a diffuse cytoplasmic staining, whereas cells lacking BRCA1 (CEFs) show only a diffuse cytoplasmic staining. Moreover, to confirm the absence of BRCA1 in CEFs, immunoprecipitations were performed with cellular extracts obtained sequentially with (i) a mild Triton-containing buffer (HNTG) that extracts cytosolic proteins and (ii) subsequently with a harsher SDS-containing buffer (RIPA) (Fig. 1B). Typically, these studies confirm that BRCA1 is present only in the nuclear fraction and not in the cytoplasm of HBL100 and MCF-7 cells. In addition, immunoreactivity against BRCA1 is not observed CEFs, confirming the absence of BRCA1 in these cells.

Is BRCA1 a granin?

The discovery of a granin sequence in BRCA1 suggested that BRCA1 and BRCA2 might be secretory proteins (Jensen et al., 1996a). Granins are a family of highly variable proteins that share a 10 amino acid motif and participate in secretory pathways (Ozawa and Takata, 1995). Interestingly, using polyclonal antibodies detected a 190 kDa in cell lysates and a 180 kDa protein from baculovirus lysates expressing the recombinant protein rather than the typical 220 kDa protein (Table 1). Furthermore, the majority of the reacting species localized to the membrane fraction and a small amount to the cytoplasm. The staining of primary human mammary epithelial cells revealed a granular pattern in both nucleus and Golgi complex (Jensen et al., 1996a). Later, using confocal microscopy Coene et al. suggested that the nuclear dot pattern represents cross-sections of cytoplasmic invaginations and that BRCA1 was mostly perinuclear (Coene et al., 1997). Although antibodies cross-reactivity may explain Golgi staining, additional evidence for BRCA1 being secreted is still lacking.

Several observations, following this initial publication, rebut the idea that BRCA1 is a granin. That includes the fact that the antibodies (Table 1) used in the

Table 1. α-BRCA1 antibodies.

ANTIBODY	BRCA1 EPITOPE[a]	USES	LOCALIZATION (REFERENCE)[b]	PROTEIN SIZE[c] AND OTHER COMMENTS
α-BRCA1	(G) 762-1315	IP[d], IB, IF	Nuclear in normal cells and cells from tumors other than breast and ovary and cytoplasmic in breast and ovarian cancer cells (Chen et al., 1995a,b).	220 kDa (Chen et al., 1995a,b). Mouse polyclonal
α-BRCA1	860-881	IF, IB	Cytoplasmic tube-like invaginations of the cytoplasm in both normal and breast cancer cells (Coene et al., 1997).	190, 220 and 240 kDa (Coene et al., 1997). Rabbit polyclonal.
α-BRCA1	1848-1863	IF, IB	Cytoplasmic tube-like invaginations of the cytoplasm in both normal and breast cancer cells (Coene et al., 1997).	190, 220 and 240 kDa (Coene et al., 1997). Rabbit polyclonal.
α-BRCA1 BgI	(G) 341-748	IP	n.s.[e]	220 kDa (Chen et al., 1995). Mouse polyclonal.
α-BRCA1N	(G) 1-302	IP		220 kDa (Chen et al., 1996). Mouse polyclonal.
A	2-20	IB, IP	Nuclear and some cytoplasmic in fractionation experiments (Ruffner and Verma, 1997).	220 kDa (Ruffner and Verma, 1997; Wilson et al., 1999). Rabbit polyclonal.
A19	1847-1863	IP, IB	Nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996).	220 kDa (Scully et al., 1996). Rabbit polyclonal.
AP11	(G) 1313-1863	IP, IB, IF	n.s.	n.s. Mouse monoclonal.
AP12	(G) 1313-1863	IP, IB, IF	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
AP16	(G) 1313-1863	IP, IB, IF	nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996; Jensen et al., 1996b). Variable in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	220 kDa (Scully et al., 1996; Wilson et al. 1999). Mouse monoclonal.
B	70-89			220 kDa (Ruffner and Verma, 1997). Rabbit polyclonal.
B112	(G) 2-355	IF, IB, IP	Diffuse nuclear staining and absent from nucleoli (Wilson et al., 1997).	230 kDa (Wilson et al., 1997); 220 kDa (Wilson et al., 1999). Rabbit polyclonal.
BPA-1	8-475	IB	n.s.	220 kDa (Thomas et al., 1996). Rabbit polyclonal.
BPA-2	1293-1863	IB, IF	Nuclear dot pattern both in normal and cancer cell lines (Thomas et al., 1996).	220 kDa (Thomas et al., 1996). Rabbit polyclonal
BR64		IF	Nuclear dot pattern (Jensen et al., 1998).	220 kDa (Jensen et al., 1998). Rabbit monoclonal, available through Upstate Biotechnology.
C	768-793	IB, IP, IF	n.s.	220 kDa (Ruffner and Verma, 1997; Wilson et al., 1999). Rabbit polyclonal.
C-19	1844-1863	IP	n.s.	180-190 kDa (Jensen et al., 1996a)[f]. Rabbit polyclonal.
C-20	1843-1862	IP, IF, IB,	Predominantly granular cytoplasmic, with nuclear and Golgi staining (Jensen et al., 1996a). Nuclear and associated with the centrosome during mitosis (Hsu and White, 1998). Nuclear (Thakur et al., 1997; Wilson et al., 1999).	220 kDa (Chen et al., 1995; Thomas et al., 1996; Thakur et al., 1997; Wilson et al., 1999); 180-190 kDa (Jensen et al., 1996a,b)[f]; 230 kDa (Wilson et al., 1996); 185 kDa (Gudas et al., 1995). Rabbit polyclonal, available through Santa Cruz Biotechnology. Cross-reacts with EGFR and HER2 (Wilson et al., 1996).
D	1847-1863	IB, IP	Nuclear and some cytoplasmic in fractionation experiments (Ruffner and Verma, 1997).	220 kDa (Ruffner and Verma, 1997). Rabbit polyclonal
D-20	1-20	IB, IF	n.s.	190 kDa (Jensen et al., 1996a); 220 kDa (Wilson et al., 1999). Rabbit polyclonal, available through Santa Cruz Biotechnology. Does not cross-react with EGFR and HER2 (Wilson et al., 1996).

continued

GLK2	1839-1863	IF	Cytoplasmic in cell lines. Cytoplasm or no signal in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	Mouse monoclonal.
I-20	1823-1842	IB	n.d.[g]	230 kDa (Wilson et al., 1996). Rabbit polyclonal, available through Santa Cruz Biotechnology. Does not cross-react with EGFR and HER2 (Wilson et al., 1996).
Ki-8	903-919	IP, IB	Nuclear in fractionation experiments (Zhang et al., 1997).	215 kDa (Zhang et al., 1997). Mouse monoclonal.
M-20	mouse BRCA1 C-terminus	IB, IF	n.d.	215 kDa (Zhang et al., 1997). Goat polyclonal, available through Santa Cruz Biotechnology.
MS110	(G) 1-304	IP, IB, IF	Nuclear dot pattern both in normal and cancer cell lines (Scully et al., 1996, 1997b; Jensen et al., 1996b; Hsu and White, 1998; Wilson et al., 1999). Variable in paraffin-embedded cell pellets depending on conditions[h] used (Jensen et al., 1996b).	220 kDa (Scully et al., 1996a; Hsu and White, 1998; Scully et al., 1997b; Wilson et al., 1999). Mouse monoclonal, available as Ab-1 from Oncogene Research Products.
MS13	(G) 1-304	IF, IP, IB	Nuclear dot pattern both in normal and cancer cell lines. Variable in paraffin-embedded cell pellets depending on conditions used (Scully et al., 1996, 1997b; Jensen et al., 1996b)	220 kDa (Scully et al., 1996, 1997b; Wilson et al., 1999). Mouse monoclonal, available as Ab-2 from Oncogene Research Products.
N25	1-25	IF	Cytoplasmic in cell lines. Cytoplasm or no signal in paraffin-embedded cell pellets depending on conditions used (Jensen et al., 1996b).	Mouse monoclonal.
SD112	(G) 758-1313	IP, IB	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
SD118	(G) 758-1313	IP, IB, IF	n.s.	220 kDa (Wilson et al., 1999). Mouse monoclonal.
SD123	(G) 758-1313	IP, IB, IF	n.s.	220 kDa (Chen et al., 1998; Wilson et al., 1999). Mouse monoclonal.
SG11	(G) 1847-1863	IF, IP	Nuclear dot pattern both in normal and cancer cell lines. Variable in paraffin-embedded cell pellets depending on conditions used (Scully et al., 1996; Jensen et al., 1996b).	Mouse monoclonal, available as Ab-3 from Oncogene Research Products.
ZB1	(G) 13-75	IB, IP	Nuclear with some amount cytoplasmic in fractionation experiments (Aprelikova et al., 1996).	220 kDa (Aprelikova et al., 1996). Rabbit polyclonal.
6B4	(G) 341-748	IP, IB		220 kDa (Chen et al., 1996a; Aprelikova et al., 1996). Mouse monoclonal.
17F8	(G) 762-1315	IP, IB, IF	Nuclear at lower (< 3 μg/ml) concentrations of antibody (Wilson et al., 1999).	220 kDa (Wilson et al., 1999). Mouse monoclonal, available through GeneTex.
24G11		IP, IB	n.d.	220 kDa (Chen et al., 1995b). Mouse monoclonal.
113	673-1365	IP, IB, IF	Nuclear (Wilson et al., 1999).	220 kDa (Wilson et al., 1999). Rabbit polyclonal.
115	673-1365	IP, IB, IF		220 kDa (Wilson et al., 1999). Rabbit polyclonal.
579	903-919	IP, IB	n.d.	215 kDa (Zhang et al., 1997). Rabbit polyclonal.

The references in this table are not extensive. Due to space limitations we have focused on the initial papers. A direct comparison of several monoclonal and polyclonal antibodies has been recently published (Wilson et al., 1999). [a]: all the antibodies have been raised against human BRCA1 epitopes unless otherwise stated. (G): antibody was raised against a GST-fusion protein. The remaining antibodies were raised using synthetic or recombinant peptides. [b]: In this table we have chosen to be conservative and not to consider experiments reported but not shown in the original papers. [c]: Although many antibodies described here showed several reactive bands, the size shown is that of the reactive species considered by the authors to represent the full length BRCA1. [d]: The uses described here were the ones reported in the original. We have not considered experiments reported but not shown. IP: immunoprecipitation; IF: immunofluorescence; IB, immunoblots. [e]: Not shown. [f]: 190 kDa species was detected from cell lysates and the 180 kDa species was detected from expressing the recombinant protein in baculovirus. [g]: Not done. [h]: Conditions vary in fixatives (neutral or alcoholic formalin) and in heat-induced epitope retrieval (no treatment; microwave or pressure cooker treatment).

majority of the experiments cross-reacts with human EGF Receptor and HER2, tyrosine kinase receptors that are frequently amplified in human breast and ovarian cancers (Wilson et al., 1996). Secondly, the statistical significance of the presence of the granin sequence in BRCA1 has been challenged, particularly allowing for a substitution in one of the motif's invariant position found in rat, dog and mouse Brca1 (Bradley and Sharan,

1996; Koonin et al., 1996). In addition, an alternative splice form of BRCA2 was found lacking the granin sequence, and shown by linkage analysis not to predispose to disease but to be a benign polymorphism (Mazoyer et al., 1996). Thirdly, the concept of BRCA1 being a tumor suppressor acting via the extracellular space has been challenged on the basis that it would be unlikely that a loss of heterozygosity in BRCA1 locus would cause a cell autonomous defect (Bradley and Sharan, 1996). In the scenario suggested by Jensen et al. (1996a), cells in breast and ovary tissue carrying no functional BRCA1 (after loss of hetero-zygosity) would still be bathed by the extracellular milieu containing BRCA1 secreted by the neighboring cells, making it unlikely to explain tumor initiation. To date, there has been no independent corroboration to the notion that BRCA1 is secreted.

The controversy resolved

The characterization of the putative nuclear localization signals (NLS) found in BRCA1 has been a key point to establish it as a nuclear protein (Miki et al., 1994). Two groups have identified slightly different NLSs [^{503}KRKRRP^{508}, ^{606}PKKNRLRRKS^{615} and ^{651}KKKKYN^{656} (Chen et al., 1996b); ^{501}KLKRK RR^{507} and ^{607}KKNRLRRK^{614} (Thakur et al., 1997)]. Chen et al. (1996b) reported that NLSs 503-508 and NLS 606-615, but not NLS 651-656, are crucial for nuclear localization as site directed mutagenesis of these sites result in cytoplasmic localization of BRCA1. Further studies have found that *BRCA1* interacts with the Importin-α subunit of the nuclear transport signal receptor. Thakur et al. (1997) also found that NLS 501-507 is critical for nuclear localization whereas deletion mutants lacking NLS 607-614 are nuclear. The discrepancy may be explained by the different mutations employed (site-directed mutagenesis versus deletion mutants). In any event, the characterization of the NLSs supports the idea that BRCA1 is a nuclear protein.

Thomas et al. (1996) have developed different antibodies (Table 1) and confirmed the nuclear localization of the 220 kDa *BRCA1* in both normal and cancer cell lines through immunofluorescence and biochemical fractionation. Moreover, Wilson et al. (1997) confirmed the nuclear staining using a panel of overexpressed epitope-tagged BRCA1 and biochemical fractionation of cells overexpressing BRCA1. Interestingly, they describe the major alternative splice variant *BRCA1* Δ11b (~ 110 kDa), that lacks the NLSs and localizes preferentially to the cytoplasm. This variant might be responsible for cytoplasmic staining in immunofluorescence since it conserves the N- and C-terminal epitopes against which the majority of the antibodies have been raised against. Even in the case of BRCA1 Δ11b variant, no evidence was found of staining in Golgi or endoplasmic reticulum (Wilson et al., 1997). Other groups raised additional antibodies (Table 1) used

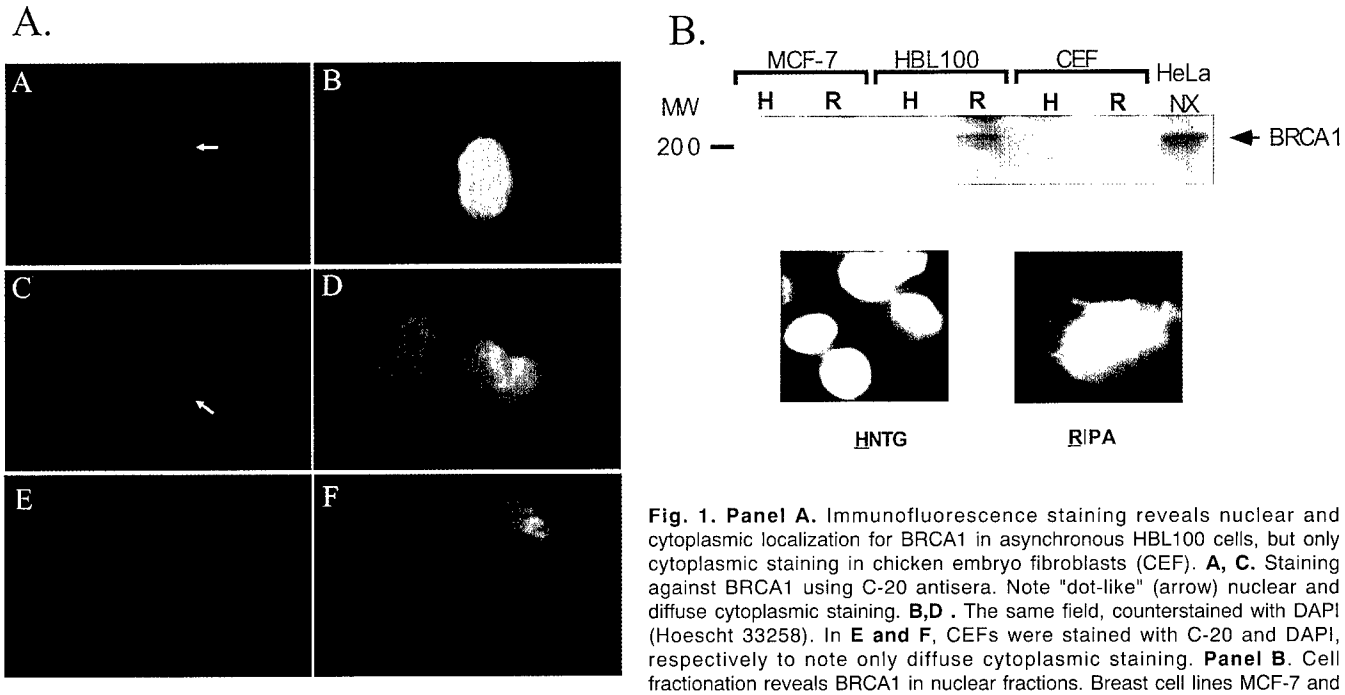

Fig. 1. Panel A. Immunofluorescence staining reveals nuclear and cytoplasmic localization for BRCA1 in asynchronous HBL100 cells, but only cytoplasmic staining in chicken embryo fibroblasts (CEF). **A, C.** Staining against BRCA1 using C-20 antisera. Note "dot-like" (arrow) nuclear and diffuse cytoplasmic staining. **B,D .** The same field, counterstained with DAPI (Hoescht 33258). In **E and F**, CEFs were stained with C-20 and DAPI, respectively to note only diffuse cytoplasmic staining. **Panel B**. Cell fractionation reveals BRCA1 in nuclear fractions. Breast cell lines MCF-7 and HBL100, and CEFs were lysed in 1% Triton-containing HNTG buffer, which is insufficient to lyse nuclear membranes or SDS-containing RIPA buffer, which readily lyses nuclear membranes (see lower DAPI panels). Detergent extracts from HNTG, RIPA, or high salt nuclear extracts (NX) were subsequently immunoprecipitated with C-20 antiserum and further immonoblotted with the same antiserum. BRCA1 was detected with HRP-conjugated secondary antibody.

for biochemical fractionation (Aprelikova et al., 1996; Thomas et al., 1996; Ruffner and Verma, 1997; Zhang et al., 1997) and immunoperoxidase staining (Rao et al., 1996) and have come to the conclusion that *BRCA1* is a nuclear protein with a molecular mass of 215-240 kDa.

Recently, Wilson et al. (1999) have undertaken an important task of comparing several different antibodies derived both from different laboratories and commercial sources in a variety of situations and consistently showed that BRCA1 is nuclear and attribute some early findings of cytoplasmic mislocalization to high concentrations of antibody used (see also Fig. 1). This study is the more comprehensive panel of antibodies tested so far and presents a compelling argument for a nuclear localization of BRCA1. More importantly, they present a series of biochemical fractionations using different antibodies to demonstrate nuclear localization. Table 1 in Wilson et al. (1999) summarizes the panel of antibodies, techniques and results obtained in parallel experiments. This table, used in conjunction with the Table 1 shown here, will be of special interest to the pathologist since it deals in detail with antibodies and conditions for immunohistochemistry in tissue blocks.

A function for BRCA1 in spindle checkpoints?

Hsu and White (1998) have presented interesting data suggesting the association of BRCA1 with the centrosome, more specifically with γ-tubulin during mitosis. Considering that the huge amounts of tubulin present in the cells are a potential cause of artifacts in immunoprecipitations, confirmation using other methods (in vitro binding assays or GST-fusion pull down) and other antibodies (besides MS110 and the problematic C-20) is needed before we can be certain. Importantly, it will be interesting to see if $BRCA1^{-/-}$ cell lines are prone to aberrant chromosome segregation caused by failure in spindle checkpoints.

A nuclear function?

Both genetics and cell biological data have led to the proposal that BRCA1 may be involved in DNA repair and in transcriptional regulation. BRCA1 contains a transcriptional activation domain localized to its C-terminus (Chapman and Verma, 1996; Monteiro et al., 1996). Interestingly, introduction of disease-predisposing mutations disrupted transcriptional activation (Chapman and Verma, 1996; Monteiro et al., 1996) while benign polymorphisms did not (Monteiro et al., 1997). In support of the proposed role in transcriptional regulation, BRCA1 has been found to be associated with the RNA polymerase II holoenzyme, through RNA helicase A (Scully et al., 1997a; Anderson et al., 1998) and to act as a coactivator for p53-mediated gene expression (Somasundaram et al., 1997; Ouchi et al., 1998; Zhang et al., 1998).

Co-localization studies by Scully et al. (1997b) have also uncovered an interaction of BRCA1 with Rad51, the homolog of bacterial RecA, suggesting a role for BRCA1 in DNA repair. In addition, DNA damage induces changes in BRCA1 subnuclear location and phosphorylation strengthening the idea that *BRCA1* is involved in DNA repair (Scully et al., 1997c; Thomas et al., 1997). Additional findings support the notion of BRCA1 may act in DNA repair, including the fact that blastocysts from $Brca1^{-/-}$ mice are more sensitive to DNA damage and tend to accumulate chromosomal abnormalities (Shen et al., 1998). Secondly, *BRCA1* has been found to be in a complex with BRCA2 (Chen et al., 1998), the product of the other major breast and ovarian cancer susceptibility gene that has also been shown to be involved in DNA repair (Connor et al., 1997; Sharan et al., 1997; Patel et al., 1998). Interestingly, a recent finding that BRCA1 is required for transcription-coupled repair in murine cells indicates that the above hypotheses for BRCA1 function are not mutually exclusive (Gowen et al., 1998).

BRCA1-Interacting proteins are nuclear

Screening methods, such as the yeast two-hybrid system, which are unbiased for proteins in any particular cellular compartment, have revealed the interaction of BRCA1 with two previously characterized proteins, c-Myc (Wang et al., 1998), a proto-oncogene that functions as a transcription factor and, CtIP a protein implicated in the CtBP pathway of transcriptional repression that was independently cloned by two laboratories (Wong et al., 1998; Yu et al., 1998). Both

Table 2. BRCA1-interacting proteins.

INTERACTING PROTEIN	BRCA1 BINDING SITE	LOCATION OF INTERACTING PROTEIN	FUNCTION OF INTERACTING PROTEIN	REFERENCE
BAP1	RING finger	nuclear	ubiquitin hydrolase	Jensen et al., 1998
BARD1	RING finger	nuclear	unknown, repair (?)	Wu et al., 1996
BRCA2	1314-1863	nuclear	transcription (?), repair	Chen at al., 1998
c-Myc	433-511	nuclear	transcription	Wang et al., 1998
CtiP	1651-1863	nuclear	transcription	Wong et al., 1998; Yu et al., 1998
γ-tubulin	n.d.	cytoplasm	cytoskeletal	Hsu et al., 1998
Importin-α	NLSs	nuclear/cytoplasm	nuclear import	Chen et al., 1996b
p53	224-500	nuclear	transcription	Ouchi et al., 1998; Zhang et al., 1998
RAD51	758-1064	nuclear	repair	Scully et al., 1997b
RNA helicase A	1650-1800	nuclear	transcriptional	Anderson et al., 1998

proteins are nuclear and their interaction with BRCA1 is consistent with the proposed role of BRCA1 in transcription.

Two previously unknown proteins were also found, BARD1, a protein of unknown function containing a RING finger and two BRCT domains (Wu et al., 1996) and BAP-1 a protein with ubiquitin hydrolase activity (Jensen et al., 1998). With exception of γ-tubulin, all the proteins found to interact in vivo with *BRCA1* are nuclear or participate in nuclear import (Table 2).

Conclusion

The controversy about BRCA1 is a cautionary tale for cell biologists as well as pathologists and illustrates the difficulty to unambiguously assign a location to a protein of unknown function. There is an overwhelming body of direct evidence that points to a nuclear localization and function for BRCA1 in normal and cancer cells. Similarly, there is an increasing amount of indirect evidence, both from interacting proteins and from functional studies of BRCA1, that support the data for the nuclear localization for BRCA1. It is too soon to evaluate the functional significance and relevance of the interacting proteins identified in several screening approaches. One way to assess the significance of the interaction is to show that interaction is disrupted by disease-causing mutations, which has been shown in some cases. In any case, results from interaction experiments seem to support the notion that BRCA1 is involved in repair and in transcriptional regulation.

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Functional Assay for BRCA1: Mutagenesis of the COOH-Terminal Region Reveals Critical Residues for Transcription Activation[1]

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ABSTRACT

The breast and ovarian cancer susceptibility gene product BRCA1 is a tumor suppressor, but its precise biochemical function remains unknown. The BRCA1 COOH terminus acts as a transcription activation domain, and germ-line cancer- predisposing mutations in this region abolish transcription activation, whereas benign polymorphisms do not. These results raise the possibility that loss of transcription activation by BRCA1 is crucial for oncogenesis. Therefore, identification of residues involved in transcription activation by BRCA1 will help understand why particular germ-line missense mutations are deleterious and may provide more reliable presymptomatic risk assessment.

The BRCA1 COOH terminus (amino acids 1560–1863) consists of two BRCTs preceded by a region likely to be nonglobular. We combined site-directed and random mutagenesis, followed by a functional transcription assay in yeast: (*a*) error-prone PCR-induced random mutagenesis generated eight unique missense mutations causing loss of function, six of which targeted hydrophobic residues conserved in canine, mouse, rat, and human BRCA1; (*b*) random insertion of a variable pentapeptide cassette generated 21 insertion mutants. All pentapeptide insertions NH_2-terminal to the BRCTs retained wild-type activity, whereas insertions in the BRCTs were, with few exceptions, deleterious; and (*c*) site-directed mutagenesis was used to characterize five known germ-line mutations and to perform deletion analysis of the COOH terminus. Deletion analysis revealed that the integrity of the most COOH-terminal hydrophobic cluster (I1855, L1854, and Y1853) is necessary for activity. We conclude that the integrity of the BRCT domains is crucial for transcription activation and that hydrophobic residues may be important for BRCT function. Therefore, the yeast-based assay for transcription activation can be used successfully to provide tools for structure-function analysis of BRCA1 and may form the basis of a BRCA1 functional assay.

INTRODUCTION

Individuals carrying mutations in the *BRCA1* gene have an increased risk of developing breast and ovarian cancer (1). Mutations in *BRCA1* alone account for ~45% of families with high incidence of breast cancer and up to 80% of families with both breast and ovarian cancer (2). After an extensive search, *BRCA1* was mapped to the long arm of chromosome 17 by linkage analysis (3) and was cloned by positional cloning techniques (4). Human *BRCA1* codes for a 1863-amino acid protein with no detectable similarity to known proteins, with the exception of a zinc-binding RING finger domain located in the NH_2-terminal region (4), and two BRCT[4] domains found in a variety of proteins involved in cell cycle control and DNA repair (5–7).

Recent evidence points to the involvement of BRCA1 in two basic cellular processes: DNA repair and transcriptional regulation. BRCA1 is present in a complex containing Rad51 (8) and BRCA2 (9), and DNA damage may control BRCA1 phosphorylation and subnuclear location (10, 11), strongly suggesting its involvement in the maintenance of genome integrity. Additional evidence for the role of BRCA1 in maintenance of genome integrity is provided by targeted disruption of *Brca1* in the mouse. Mouse embryos lacking *Brca1* are hypersensitive to γ-irradiation, and cells display numerical and structural chromosomal aberrations (12).

We and others have shown that the BRCA1 COOH terminus has the ability to activate transcription in mammalian and yeast cells and that the introduction of germ-line disease-associated mutations, but not benign polymorphisms, abolishes this activity (13–15). BRCA1 can be copurified with the RNA polymerase II holoenzyme, supporting the idea that BRCA1 is involved in transcription regulation (16, 17). In addition, BRCA1 causes cell cycle arrest via transactivation of $p21^{WAF1/CiP1}$ (18) and regulates p53-dependent gene expression, acting as a coactivator for p53 (19, 20). In all of these studies, the COOH-terminal region was necessary for activity. It is still not clear whether BRCA1 is a multifunctional protein with repair and transcription regulation functions or whether the role of BRCA1 in repair is mediated through transcription activation. In either case, these functions are not necessarily mutually exclusive.

The dearth of knowledge concerning the precise biochemical function of BRCA1 is a major hurdle in developing a functional test to provide reliable presymptomatic assessment of risk for breast and ovarian cancer. The available data derived from linkage analysis indicate that all mutations that cause premature termination (even relatively subtle mutations such as the deletion of 11 amino acids from the COOH terminus) will confer high risk (21). However, a considerable number of mutations result in amino acid substitutions that, in the absence of extensive population-based studies or a functional assay, do not allow assessment of risk. Two related yeast-based assays designed to characterize mutations in the BRCA1 COOH terminal region have generated results that provide an excellent correlation with genetic linkage analysis (13, 14, 22). This led us to propose the general use of a yeast-based assay to provide functional information and a more reliable risk assessment (23).

In this report, we use site-directed and random mutagenesis to generate mutations in the BRCA1 COOH terminal region that disrupt transcription activation with the intention of both defining critical residues for BRCA1 function and deriving general rules to predict the impact of a particular mutation.

MATERIALS AND METHODS

Yeast Strains. Three *Saccharomyces cerevisiae* strains were used in this study: HF7c [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3, 112, gal4–542, gal80–538, LYS::GAL1-HIS3, URA3::(GAL4 17mers)$_3$-CYC1-lacZ*]; SFY526 [*MATa, ura-3–52, his3–200, lys2–801, ade2–101, trp1–901, leu2–3,112,* can^r, *gal4–542, gal80–538, URA3::GAL1-lacZ*]

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[4] The abbreviations used are: BRCT, BRCA1 COOH terminal domain; DBD, DNA binding domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MTD, minimal transactivation domain.

(24); and EGY48 [*MATα, ura3, trp1, his3, 6 lexA* operator-*LEU2*] (25). HF7c has an *HIS3* reporter gene under the control of the *GAL1* upstream activating sequence, responsive to GAL4 transcription activation. The vectors used for expression confer growth in the absence of tryptophan (see below). The SFY526 strain has a *lacZ* reporter under the control of *GAL1* upstream activating sequence and was transformed with the GAL4 DBD fusion. EGY48 cells were cotransformed with the LexA fusion vectors and plasmid reporters of *lacZ* under the control of LexA operators (see below). If the fusion proteins activate transcription, EGY48 and SFY526 yeast transformants will produce β-galactosidase, and HF7c transformants will grow in medium lacking histidine.

Yeast Expression Constructs. The GAL4 DBD fusion of the wild-type human BRCA1 COOH terminal region (amino acids 1560–1863) was described previously (13). Alternatively, this fragment was subcloned into the yeast expression vector pLex9 (25) in-frame with the DBD of LexA. Both plasmids have *TRP1* as a selectable marker, allowing growth in the absence of tryptophan. We noticed that our previously described BRCA1 (amino acids 1560–1863) construct (13) was made with a 3′ primer lacking a termination codon. This introduces 16 exogenous amino acids to the COOH-terminal region of BRCA1. We have corrected this by using primer 24ENDT (5′-GCGGATCCTCAGTAGTGGCTGTGGGGGAT-3′). We compared both constructs and ascertained that qualitatively and quantitatively, they have the same activity (not shown).

BRCA1 deletion mutants were generated by PCR on a BRCA1 (amino acids 1560–1863) context using pcBRCA1–385 (a gift from Michael Erdos, National Human Genome Research Institute) as a template and the following primers: H1860X (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S970074, 5′-GCGGATCCTCAGGGGATCTGGGG-3′); P1856X (S9503101, S970073, 5′-GCGGATCCTCATATCAGGTAGGTGTCC-3′); I1855X (S9503101, 1855STOP, 5′-GCGGATCCTCACAGGTAGGTGTCC-3′); and L1854X (S9503101, 1854STOP, 5′-GCGGATCCTCAGTAGGTGTCCAGC-3′). Mutant Y1853X corresponds to a germ-line mutation and has been described previously (13). The constructs were sequenced to verify the mutations. The PCR products were digested with *Eco*RI and *Bam*HI and subcloned into similarly digested pGBT9 vectors. Alternatively, the PCR fragments were subcloned into a vector, pAS2–1 (Clontech), with higher expression levels. Introduction of additional mutations was made using the Quick-Change method. Briefly, a pair of primers encoding each mutation flanked by homologous sequence on each side was added to the wild-type plasmid pLex9 BRCA1 (amino acids 1560–1863) prepared in a methylation-competent strain. The plasmid was amplified using *Pfu* polymerase (one cycle at: 96°C for 30 s; 12 cycles at: 96°C for 30 s; 50°C for 1 min; and 68°C for 12 min), and *Dpn*I was added at the end of the reaction to digest the parental plasmid. The mixture was then transformed into bacteria. The following oligonucleotide primers were used: T1561I (T1561IF, 5′-CTGGAATTCGAGGGAATCCCTTACCTCGAGTCTGG-3′; T1561IR, 5′-CCAGACTCGAGGTAAGGGATTCCCTCGAATTCCAG-3′); L1564P (L1564PF, 5′-GGGTACCCCTTACCCGGAATCTGGAATCAG-3′;L1564PR,5′-CTGATTCCAGATTCCGGGTAAGGGGTACCCTC-3′); D1733G (D1733GF, 5′-GAAAAATGCTCAATGAGCATGGTTTTGAAGTCCGCGGAG-3′; D1733GR, 5′-CTCCGCGGACTTCAAAACCATGCTCATTCAGCATTTTTC-3′); G1738E (G1738EF, 5′-GAGCATGATTTTGAAGTCAGAGAAGATGTGGTTAACGGAAG-3′; G1738ER, 5′-CTTCCGTTAACCACATCTTCTCTGACTTCAAAATCATGCTC-3′); P1806A (P1806AF, 5′-GGTACCGGTGTCCACGCAATTGTGGTTGTGCAGC-3′; and P1806AR, 5′-GCTGCACAACCACAATTGCGTGGACACCGGTACC-3′).

Yeast Plasmid Reporters. Plasmid pSH18-34 (25), a kind gift of Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA), was used as a reporter in the LexA fusion assays. This vector has *lacZ* under the control of eight LexA operators, conferring low stringency of gene expression (26).

Yeast Transformation. Competent yeast cells were obtained using the yeast transformation system (Clontech) based on lithium acetate, and cells were transformed according to the manufacturer's instructions.

Filter β-Galactosidase Assay. SFY526 and EGY48 transformants (several clones for each construct) were streaked on a filter overlaid on solid medium lacking tryptophan (SFY526) or tryptophan and uracil (EGY48) and allowed to grow overnight. Cells growing on the filter were lysed by freeze/thawing in liquid nitrogen, and each filter was incubated in 2.5 ml of Z buffer (16.1 g/liter $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, and 0.246 g/l $MgSO_4{\cdot}7H_2O$, pH 7.2) containing 40 μl of X-gal solution (20 mg/ml of X-gal in *N,N*-dimethylformamide) at 30°C for up to 16 h.

Liquid β-Galactosidase Assay. Liquid assays were performed as described previously (27). At least three separate transformants were assayed, and each was performed at least in duplicate.

Growth Curves. HF7c transformants (several clones) containing different pGBT9 or pAS2 constructs were grown overnight in synthetic medium plus 2% dextrose (SD medium) lacking tryptophan. The saturated cultures were used to inoculate fresh medium lacking tryptophan or tryptophan and histidine to an initial A_{600} of 0.0002. Cultures were grown at 30°C in the shaker, and the absorbance was measured at different time intervals starting at 12 h, then every 4 h up to 36 h after inoculation.

Plasmid Recovery from Yeast Cells. EGY48 transformants were grown to saturation in liquid medium lacking uracil (but in the presence of tryptophan). Cells were harvested and treated with yeast lysis solution [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA], phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 g of acid-washed beads. The sample was vortexed for 2 min and centrifuged, and the supernatant precipitated with one-tenth volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of ethanol. Alternatively, plasmid rescue was performed as suggested by Strathern and Higgins (28).

Screening in X-gal Plates. To allow direct screening of the clones with loss of activity, EGY48 cells transformed with the mutagenized cDNAs were plated on X-gal-containing plates: 2% galactose, 1% raffinose, 80 mg/l X-gal, and 1× BU salts (1 liter of 10× BU salts: 70 g $Na_2HPO_4{\cdot}0.7H_2O$, 30 g NaH_2PO_4).

Error-prone PCR Mutagenesis. A 60-cycle PCR reaction (94°C denaturation; 55°C annealing; 72°C extension) was performed using Taq polymerase and p385-BRCA1 plasmid as a template and oligonucleotide primers (S9503101, 5′-CGGAATTCGAGGGAACCCCTTACCTG-3′; S9503098, 5′-GCGGATCCGTAGTGGCTGTGGGGGAT-3′). The PCR product was gel purified and cotransformed in an equimolar ratio with a *Nco*I-linearized wild-type pLex9 BRCA1 (amino acids 1560–1863) plasmid and pSH18-34. After transformation, cells were plated on X-gal plates and incubated for 5 days. Eighty-one white and four control blue clones were recovered and restreaked on master plates. White clones were screened again on a filter assay, and the 62 clones that were consistently white were analyzed further. Plasmid DNA was recovered from the yeast cells and transformed into *Escherichia coli*. Miniprep DNAs from each of two bacterial transformants from the 62 candidates were retransformed into yeast cells and tested again for β-galactosidase production. The BRCA1 inserts in plasmid DNAs generating white clones were subjected to direct sequencing using dye terminators.

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a technique whereby 5-amino acid insertions are introduced at random in a target protein (29). Briefly, an *E. coli* donor strain containing the target plasmid and pHT385, a conjugative delivery vector for transposon Tn*4430*, is mated with a plasmid-free *E. coli* recipient strain. By plating the mating mix simultaneously on antibiotics selecting for the recipient, the target plasmid, and Tn*4430*, transconjugants containing pHT385::target plasmid cointegrates are isolated. This cointegrate resolves rapidly *in vivo*, regenerating pHT385 and the target plasmid into which a copy of Tn*4430* has been inserted. Tn*4430* contains *Kpn*I restriction enzyme sites located 5-bp from both ends of the transposon and duplicates 5-bp of target site sequence during transposition. By digesting the target plasmid::Tn*4430* hybrid with *Kpn*I and religating the digested DNA, the bulk of the transposon is deleted to generate a target plasmid derivative containing a 15-bp insertion. If the insertion is in a protein-encoding sequence, this will result in a 5-amino acid insertion in the target protein.

Tn*4430* insertions in the COOH-terminal region of BRCA1 were identified either by genetic or physical means. In the former case, 30 separate matings were performed as detailed previously (30) using appropriate antibiotic selections and in which the target plasmid was pLex9 containing the BRCA1 COOH terminal region fused to LexA DBD. Transconjugant colonies were harvested by washing from the mating plates, and plasmid DNA was isolated from the pooled colonies. The plasmid preparations were pooled further and transformed into *Saccharomyces cerevisiae* EGY48 harboring the pSH18-34 reporter plasmid. Transformants were tested for transcription activation by replica-plating to plates containing X-gal. Plasmid DNA was recovered from white colonies and transformed into *E. coli* XL1-Blue selecting on X-gal-

Table 1 *Missense mutations leading to loss of function (PCR-mediated mutagenesis screen)*

Exon	Mutation	Dog[a]	Mouse[b]	Rat[c]	Nucleotide[d]	Base change	Comments and probable secondary structure elements[e]
16	M1652K	M	M	M	5074	T to A	Residue mutated in germ line (M1652T, M1652I).
18	K1702E	K	K	K	5223	A to G	α-Helix 2 of BRCT-N.
18	Y1703H	Y	Y	Y	5226	T to C	α-Helix 2 of BRCT-N.
18	L1705P	L	L	L	5233	T to C	Found in two independent clones. Located just after α-helix 2 of BRCT-N.
21	F1761S	F	F	F	5401	T to C	BRCT-N/BRCT-C interval.
21	F1761I	F	F	F	5400	T to A	BRCT-N/BRCT-C interval.
22	L1780P	L	L	L	5458	T to C	α-Helix 1 of BRCT-C. Hydrophobic residue conserved in BRCT superfamily. Mediates interaction between α-helix 1 and α-helix 3.
24	V1833E	V	V	V	5617	T to A	β-Strand 4 of BRCT-C. Residue mutated in germ line (V1833M) and found in two independent clones. Hydrophobic residue conserved in BRCT superfamily.

[a] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[b] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[c] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[d] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[e] According to a BRCA1 BRCT model from Zhang *et al.* (36).

containing plates. Plasmid DNA was isolated from white colonies (which contain only pLex9::BRCA1 COOH-terminal::Tn*4430*), and the insertion of Tn*4430* into the BRCA1 COOH terminal region was confirmed by restriction enzyme mapping. For the identification of Tn*4430* insertions by physical means, pooled plasmid DNA from *E. coli* consisting of the target plasmid into which Tn*4430* was inserted was digested with *Eco*RI and *Bam*HI, enzymes which liberate the BRCA1 insert but do not cut Tn*4430*. This digestion of pooled plasmid DNA generates four fragments: the pLex9 vector backbone, the pLex9 vector containing Tn*4330* insertions, the BRCA1 COOH-terminal fragment, and the BRCA1 COOH terminal fragment containing Tn*4430* insertions. The latter fragment was recovered from an agarose gel and recloned in *Eco*RI-*Bam*HI-digested pLex9 to produce a library of pLex9::BRCA1 COOH terminal domain plasmids containing Tn*4430* insertions in the BRCA1 COOH terminal region. In the case of Tn*4430* insertions identified by either genetic or physical means, following further restriction mapping the bulk of Tn*4430* was deleted from selected clones by digestion with *Kpn*I and religation. The positions of the 15-bp insertions were determined by sequence analysis. Twenty-one plasmids harboring the BRCA1 COOH terminal region with 15-bp insertions were analyzed for transcription activation in *S. cerevisiae* EGY48 containing pSH18–34.

Western Blots. Yeast cells were grown in selective media to saturation, and A_{600} was measured. Cells were harvested and lysed in cracking buffer [8 M urea, 5% SDS, 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, and 0.4 mg/ml bromphenol blue; used 100 μl/7.5 total A_{600}] containing protease inhibitors. The samples were boiled and separated on a 10% SDS-PAGE. The gel was electroblotted on a wet apparatus to a polyvinylidene difluoride membrane. The blots were blocked overnight with 5% skim milk using TBS-Tween and incubated with the α-pLexA (for LexA constructs) or α-GAL4 DBD (for GAL4 constructs) monoclonal antibodies (Clontech) using 0.5% BSA in TBS-Tween. After four washes, the blot was incubated with the α-mouse IgG horseradish peroxidase conjugate in 1% skim milk in TBS-Tween. The blots were developed using an enhanced chemiluminescent reagent (DuPont NEN, Boston, MA).

RESULTS

Germ-Line Mutations. We analyzed missense mutations occurring in the region from amino acid 1560 to amino acid 1863 described in the Breast Cancer Information Core[5] database. To date, 63 missense variants representing mutations in 55 different residues have been documented, most of which have not been characterized either as disease-associated or as benign polymorphisms. Only four missense mutations have been either confirmed or considered very likely to be associated with disease: A1708E (31–33), P1749R (34), R1751Q (33), and M1775R (4, 31, 35). Three of these four mutations target hydrophobic residues that are conserved in canine, mouse, and rat Brca1. Amino acid composition analysis of this region reveals that only 39% of the residues are hydrophobic. Thus, although the number of characterized mutations is limited, it suggests a preference for loss-of-function mutations to target hydrophobic residues.

Mutagenesis Strategies. To shed light on the critical residues and regions necessary for function, we used four complementary strategies: (*a*) error-prone PCR mutagenesis followed by a screen for loss of function; (*b*) pentapeptide insertion mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus.

Error-prone PCR Mutagenesis Reveals Critical Residues for Activation. Approximately 10^5 yeast clones were screened for loss of transcription activation function. Sixty-two clones were isolated that had lost activity, most of which contained small insertions or deletions causing frameshift mutations and premature termination of the BRCA1 protein, as subsequently confirmed by SDS-PAGE and Western blot analysis (not shown). Two independent clones displayed the same nonsense mutation (Y1769X). Four clones had two mutations (E1660G/M1689K, K1727R/L1786P, S1722P/N1774Y, and S1715N/Q1811L), limiting their further characterization. The 10 remaining clones each had a single missense mutation (one clone also had a silent mutation) and corresponded to eight distinct mutations (Table 1). Interestingly, the screen revealed that hydrophobic residues were the major targets of mutation (six of eight). Furthermore, all of the targeted residues are perfectly conserved in canine, mouse, and rat Brca1 (Table 1). Even conservative mutations may not be well accepted in residues that are perfectly conserved in all species. This is illustrated by mutation F1761I, where a smaller hydrophobic residue is not tolerated in place of a bulkier one. Loss-of-function mutations were located primarily in the BRCT domains. In particular, mutations that occur in BRCT-C [the most COOH-terminal BRCT (amino acids 1756–1855); BRCT-N (amino acids 1649–1736) is located NH_2-terminally to BRCT-C] are in residues that constitute the hydrophobic clusters conserved in the BRCT superfamily. Western blot analysis of the mutant clones (three independent clones of each) revealed that all of the mutants were expressed at levels comparable with the wild type, ruling out the possibility that loss of function was attributable to instability of the protein (Fig. 1). It is important to stress, however, that protein levels are relatively variable in different yeast clones carrying the same constructs and should only be taken as a rough estimate.

Pentapeptide Scanning Mutagenesis Reveals Buried Regions Necessary for Activity. The BRCA1 COOH terminal region was subjected to pentapeptide scanning mutagenesis in which a variable, 5-amino acid cassette was introduced at random. The resulting set of mutated proteins included mutants that displayed complete loss of activity, mutants with reduced activity, and mutants with similar or

[5] Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/.

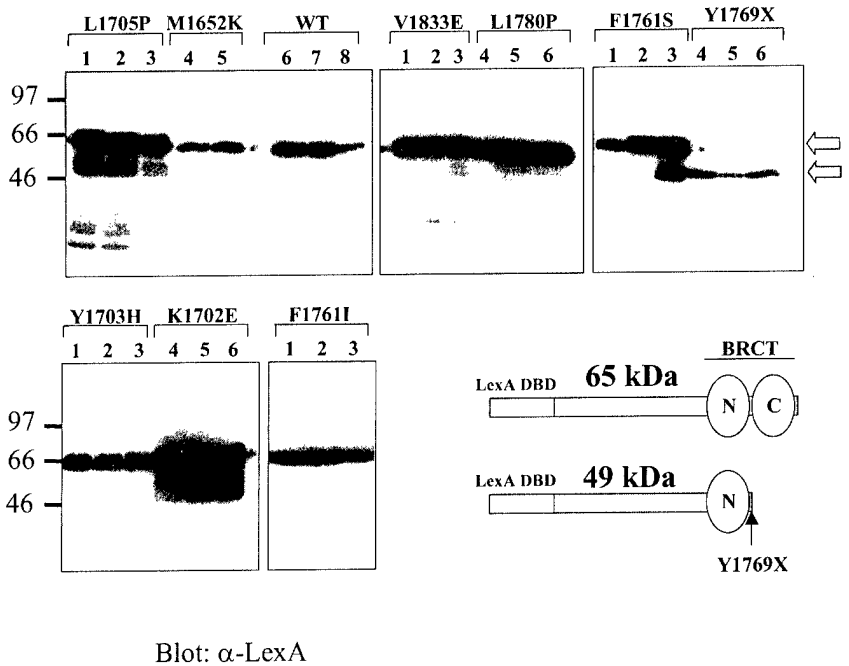

Fig. 1. Expression levels of loss-of-function BRCA1 mutants. Cell lysates containing comparable cell numbers were separated on SDS-PAGE. At least two independent transformants were assayed for each mutant to control for clonal variation. LexA fusion mutant proteins expressed in yeast were detected by Western blot using a monoclonal α-LexA antibody. A schematic representation of the fusion proteins bearing missense mutations [*white arrow*, M_r 65,000 (*65 kDa*)] and a nonsense mutation [*gray arrow*, M_r 49,000 (*49 kDa*)] is shown. Note that mutation Y1769X disrupts the BRCT-C but retains BRCT-N.

higher activity than wild type. Table 2 groups the insertions by location: the first group includes mutations in the region NH_2-terminal to the BRCT domains (amino acids 1560–1649); the second group contains mutations in BRCT-N; and the third group includes mutations in the intervening region between BRCT-N and BRCT-C. The last group includes mutations in BRCT-C. None of the insertions NH_2-terminal to the BRCT domains had a negative effect on transcription activation. Also, insertions in the interval between the BRCT domains or at its boundary (1723RGTPI) had generally less drastic effects. In contrast, all insertions within BRCT-N and several within BRCT-C had a more severe effect. It is clear that BRCT-C tolerates insertions better (only three of five showed loss of activity) than BRCT-N (all mutations reduced activity with six of seven showing drastic impairment). The difficulty in predicting the outcome of mutations can be well exemplified by mutations 1824GGTPI and 1822GVPLH. Both of these mutations target residues at the end of BRCT-C α-helix 2, do not change the net charge of the protein, and are only two residues apart. However, 1822GVPLH has ~6% of the wild-type activity, whereas 1824GGTPI has an activity ~80% higher than wild type. Interestingly, the 1793GVPLK insertion increased transcriptional activation ~4-fold, suggesting that this region of BRCA1 may directly contact a component of the transcription machinery. The pentapeptide mutagenesis results demonstrated that, in addition to substitution mutations, insertion mutagenesis in the COOH-terminal region, particularly in the BRCT domains, can profoundly alter transcriptional activity by BRCA1.

Table 2 *Transcriptional activity of insertion mutants*

Pentapeptide insertion	Miller units[a]	Probable secondary structure element[b]
Empty vector	4.1 ± 3.0	
Wild-type	99.9 ± 14.1	
1571SEGYP	**98.2 ± 98.2**	**Unknown**
1578PSGVP	**120.1 ± 52.6**	**Unknown**
1602PQGVP	**99.3 ± 15.9**	**Unknown**
1620DRGTP	**127.1 ± 11.0**	**Unknown**
1625NGVPH	**81.7 ± 8.2**	**Unknown**
1627MGVPP	**94.4 ± 6.8**	**Unknown**
1665stop	1.6 ± 0.1	α-Helix 1 of BRCT-N
1676RGTPL	2.5 ± 0.2	β-Strand 2 of BRCT-N
1678RGTPN	0.7 ± 0.2	β-Strand 2 boundary of BRCT-N
1695GVPQF	4.3 ± 1.1	β-Strand 3/α-helix 2 loop of BRCT-N
1709GGTPG	1.0 ± 0.7	α-Helix 2/β-strand 4 loop of BRCT-N
1717WGTPF	2.1 ± 0.4	α-Helix 3 of BRCT-N
1723RGTPI	36.5 ± 15.0	α-Helix 3 boundary of BRCT-N
1724GVPLK	10.4 ± 2.5	BRCT-N/BRCT-C interval
1730GVPLN	57.7 ± 7.2	BRCT-N/BRCT-C interval
1737GVPLR	1.0 ± 0.5	BRCT-N/BRCT-C interval
1769GGYPY	11.7 ± 11.1	β-Strand 1/α-helix 1 loop of BRCT-C
1780GVPQL	0.8 ± 0.3	α-Helix 1 of BRCT-C
1793GVPLK	**372.9 ± 113.8**	**β-Strand 2/β-strand 3 turn of BRCT-C**
1822GVPLH	5.7 ± 0.5	α-Helix 2 of BRCT-C
1824GGTPI	**178.0 ± 34.4**	**α-Helix 2 boundary of BRCT-C**

[a] Mutants in bold displayed activity equal to or higher than wild type.
[b] According to a BRCA1 BRCT model from Zhang *et al.* (36).

Characterization of Germ-Line Mutations. To assess the activity of variants that have already been documented but not characterized, we decided to introduce a set of mutations and assay for transcription activation in yeast (Table 3). Mutations T1561I and L1564P are both located in the region preceding the BRCT domains and displayed wild-type activity. L1564P was expected to be a polymorphism because proline is the residue found in the rat Brca1 sequence. The three remaining variants are localized to the BRCT domains. Two variants, D1733G and P1806A, displayed wild-type activity and are suggested to be benign polymorphisms. D1733G introduces a glycine that probably does not affect BRCT structure. P1806A involves a conservative change, and it is important to note that the rat Brca1 sequence has leucine in that position. Only one of the variants tested, G1738E, displayed a loss of function phenotype. Thus, we propose that G1738E is a disease-predisposing variant.

Deletion Mutants of COOH-Terminal Residues Define the Minimal Transactivation Domain (MTD). A construct carrying the germ-line mutation Y1853X does not have detectable transcriptional activity in the context of a GAL4 DBD fusion of the BRCA1 COOH terminus (amino acids 1560–1863; Refs. 13 and 15). A construct containing amino acids 1760–1863 can be considered the MTD, defining I1760 as a 5′ border of this domain (13, 15). Thus, the NH_2-terminal border of the MTD coincides closely with the NH_2-

Table 3 *Transcriptional activity of human BRCA1 unclassified variants (amino acids 1560–1863)*

Exon	Mutation	Activity[a]	Dog[b]	Mouse[c]	Rat[d]	Nucleotide[e]	Base change	Probable secondary structure element[f]	Reference
16	T1561I	+	A	T	T	4801	C to T	Unknown	Durocher *et al.* (41)
16	L1564P	+	L	L	P	4810	T to C	Unknown	BIC[g]
20	D1733G	+	D	E	E	5317	A to G	BRCT-N/BRCT-C interval	BIC
20	G1738E	−	G	G	G	5332	G to A	BRCT-N/BRCT-C interval	BIC
23	P1806A	+	P	P	L	5535	C to G	β-Strand 2/β-strand 3 loop of BRCT-C	BIC

[a] At least 10 independent clones were assayed and scored 8 h after the addition of X-gal. +, blue with same intensity as wild-type control. −, white, similar to two (F1761S and Y1769X) loss-of-function controls.
[b] Amino acid corresponds to the predicted translation from canine *Brca1* cDNA deposited in GenBank accession no. U50709.
[c] Amino acid corresponds to the predicted translation from murine *Brca1* cDNA deposited in GenBank accession no. U68174.
[d] Amino acid corresponds to the predicted translation from rat *Brca1* cDNA deposited in GenBank accession no. AF036760.
[e] Nucleotide numbering corresponds to human *BRCA1* cDNA deposited in GenBank accession no. U14680.
[f] According to a BRCA1 BRCT model from Zhang *et al.* (36).
[g] Breast Cancer Information Core.

terminal border of BRCT-C (I1760 is the first conserved hydrophobic residue in the BRCT superfamily). To identify the COOH-terminal border of the MTD, several deletion mutants were made in the amino acids 1560–1863 context and assayed for their ability to activate transcription in yeast. Fig. 2 shows the several deletion mutants analyzed aligned to mouse, rat, dog, and human BRCA1 wild-type sequences. Mutant H1860X introduces a stop codon but maintains all of the conserved amino acids in canine and human BRCA1. P1856X maintains the hydrophobic residues, which are conserved in all of the BRCT domains described in several species. I1855X and L1854X delete one and two conserved hydrophobic residues, respectively. Y1853X is a mutation found in the germ-line of breast and ovarian cancer patients in high-risk families (21). These constructs were transformed into SFY526 and HF7c and analyzed for their ability to activate different reporters (Fig. 2*b*). Activity comparable with the wild-type was obtained with mutants H1860X and P1856X. However, mutations that disrupted the conserved hydrophobic residues (I1855X and L1854X) at the end of the BRCT domain abolished activity. Therefore, we define the MTD in BRCA1 as amino acids 1760–1855. To determine whether the loss of activity by the mutants correlated with the stability of the protein, yeast cells were transformed with the same mutated alleles in a vector conferring high expression (pAS2-1). Transcriptional activity using these constructs (in pAS2-1 backbone) was measured, and results were similar with I1855X showing some residual activity. Expression was highly variable, and mutants were in general expressed at lower levels than wild type (Fig. 2*c*). There was no correlation between loss of activity and lower levels of expression because the transcriptionally active mutant H1860X was expressed at levels lower or comparable with transcriptionally inactive mutants I1855X and Y1853X (Fig. 2*c*).

DISCUSSION

In this report, we describe an extensive mutagenesis analysis of the BRCA1 COOH terminal region and partly define the critical requirements for transcriptional activity by BRCA1. Four complementary strategies were used: (*a*) error-prone PCR mutagenesis, followed by a screen for loss of function; (*b*) pentapeptide scanning mutagenesis; (*c*) site-directed mutagenesis to analyze documented mutations; and (*d*) deletion analysis of the COOH terminus. Our results support the notion that there are no particular hot spots for loss-of-function mutations, but rather that these mutations are scattered throughout the coding sequence. Nevertheless, we were able to identify preferential sites critical for activation. An overview of the mutations and their effects is presented in Fig. 3. We discuss the general conclusion of each strategy and then we analyze the possible structural outcome of the mutations based on the crystal structure of XRCC1 BRCT (36).

Error-prone PCR Mutagenesis. Eight distinct BRCA1 mutations were recovered that resulted in loss of transcription activation function. In the course of the screening procedure, many additional clones that displayed a light blue color were noted and were probably mutants with reduced function, but only clones with complete loss of function were analyzed further. No PCR-generated mutations were found in the region external to the BRCT domains, although this constitutes approximately one-third of the tested sequence, indicating a preference for mutations that affect transcription activation to occur in the BRCT domains (Fig. 3).

Six of eight unique PCR-generated mutations were in hydrophobic residues conserved in human, canine, mouse, and rat Brca1 (6, 7), supporting the notion that hydrophobic residues are important for the stability of the BRCT domains and BRCA1 function *in vivo*.

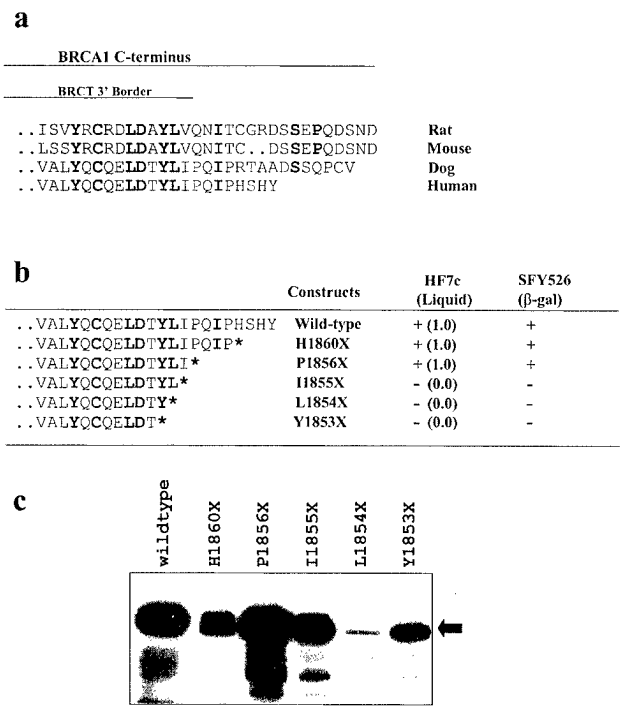

Fig. 2. Deletion analysis of the COOH-terminal region. *a*, alignment of the wild-type sequences of the COOH terminus of rat, mouse, dog, and human BRCA1. Amino acids in *bold* represent conserved residues. *Shaded area*, residues at the 3′ border of the BRCT-C domain. *b*, transcriptional activity of GAL4 DBD fusion deletion constructs, made in the context of BRCA1 amino acids 1560–1863. *S. cerevisiae* (HF7c) carrying the indicated fusion proteins were assayed for growth in the absence of tryptophan and histidine in liquid medium. Activity relative to cells growing in medium lacking tryptophan alone after 36 h is shown in *parentheses*. Filter β-galactosidase assays for SFY526 were scored at 12 h after X-gal addition. At least four independent clones were assayed for each construct. *c*, Western blot showing levels of protein expression of the different constructs (*black arrow*) detected by a α-GAL4-DBD monoclonal antibody.

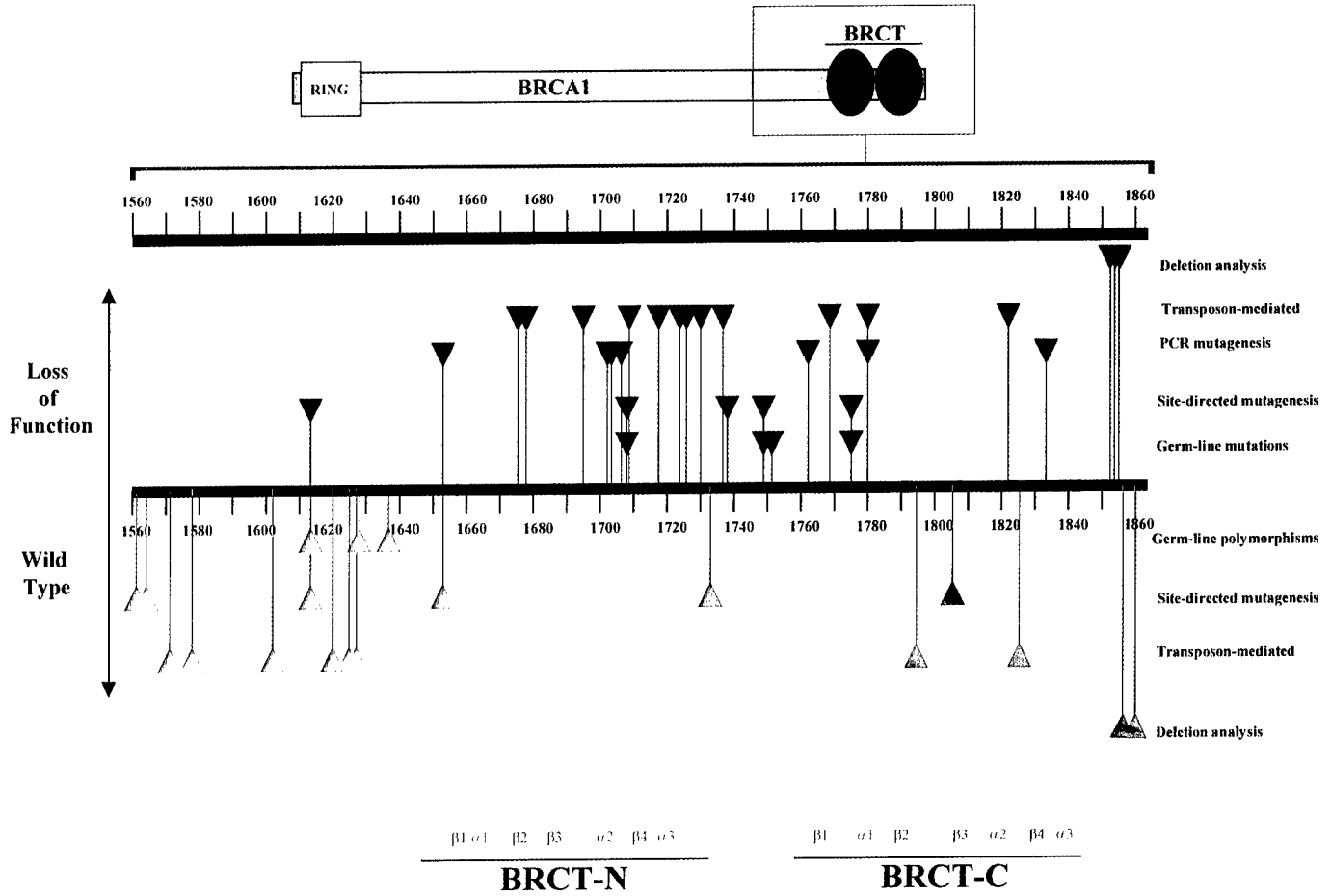

Fig. 3. Domain structure of the BRCA1 COOH terminal region (amino acids 1560–1863) and characterized mutations. *Top panel*, a schematic representation of full-length BRCA1 protein featuring the RING domain (*yellow box*) in the NH_2-terminal region and the BRCT domains (*red circles*) in the COOH-terminal region. The region analyzed in this study is contained in the *red box*, which is enlarged and represented in the *bottom panel*. *Purple* and *pink bars*, predicted β-strands and α-helices, respectively. Secondary structure predictions were made by Zhang *et al.* (36) based on the crystal structure of the XRCC1 BRCT domain. Mutations represented in the *upper part* (*red triangles*) result in loss of function, whereas mutations in the *lower part* (*green triangles*) result in activity equal or higher than wild type. Germ-line mutations and polymorphisms are variants defined by genetic linkage to be disease-associated and benign polymorphisms, respectively. Site-directed mutagenesis, PCR mutagenesis, transposon-mediated mutagenesis, and deletion analysis represent mutations that have been characterized by transcription activation assay in yeast to be either loss of function (*upper part*) or wild type (*lower part*).

Pentapeptide Scanning Mutagenesis. Pentapeptide scanning mutagenesis is a method by which a variable 5-amino acid cassette is introduced at random into a target protein (29, 30, 37). This approach differs from error-prone mutagenesis because clones are not selected for loss of activity but rather mutations are analyzed only after they have been generated. Therefore, mutants with gain of function, loss of function, and novel activities can be produced (30, 37). Moreover, it has been shown that insertion is essentially random (29). The results obtained are in agreement with the PCR-mediated mutagenesis in that the region NH_2-terminal to the BRCT domains (amino acids 1560–1649) seems to be more tolerant of mutation; none of six different pentapeptide insertions in this region affected transcription activation. The fact that derivatives containing insertion mutations in this region retained wild-type activity suggests that this region is nonglobular and is probably a flexible part of the COOH-terminal region without many critical secondary structure elements. In fact, the region encompassing amino acids 1524–1661 is predicted to be nonglobular (5). The pentapeptide mutagenesis results also suggest that changing the net charge of the protein does not necessarily correlate with an alteration in transcription activity, as would be expected for classical acidic activators (38), because 1793GVPLK (which adds a positive charge) shows a 4-fold increase in activity. Interestingly, only 4 of the 63 COOH-terminal germ-line variants involve nonconservative substitutions in acidic residues, thought to be important for activation, suggesting that, contrary to initial predictions, BRCA1 may not be a classical acidic activator (4). The 1793GVPLK mutation, which is hyperactive for transcription activation, may define a point of contact between the BRCA1 COOH-terminal region and the transcription machinery.

Deletion Analysis. Our analysis demonstrates that residues COOH-terminal to amino acids 1855 are dispensable for activation, consistent with the extreme evolutionary divergence of those residues (Fig. 2; Refs. 39 and 40). The results also underscore the importance of the last hydrophobic cluster in the sequence (YLI for human and canine; YLV for mouse and rat) and provide a plausible explanation for the complete loss of function (*in vitro* and *in vivo*) of Y1853X alleles.

Site-directed Mutagenesis. Only one of five germ-line mutations analyzed displayed loss of function, suggesting that a large part of variants in the COOH-terminal region will probably be benign polymorphisms, including some variants found in the BRCT domains. Very little data are available at this moment to confirm or contradict the results obtained. In particular, T1561I illustrates the difficulties involved in predicting outcome from population data. T1561I was found in one affected individual but not in control individuals (41). This could suggest that T1561I is a disease-predisposing variant. However, although found as a germ-line mutation, it was absent from

the tumor from the same patient (41), indicating that this mutation is a benign polymorphism.

Structural Basis for Effects of BRCT Domain Mutations. The COOH-terminal BRCT domain of XRCC1 consists of a four-stranded parallel β-sheet (β1–β4) surrounded by three α-helices (α1–α3; Ref. 36). The β-sheet forms the core of the structure with a pair of α-helices (α1 and α3) on one side of the β-sheet and the remaining α-helix (α2) on the other side. A model of the more COOH-terminal BRCT domain of BRCA1 has been constructed based on the crystal structure of the BRCT domain of XRCC1 (36). This model allows an interpretation of the effect of some of the mutations described in this study (Tables 1–3) on BRCT domain structure (Fig. 3).

The position of the M1652K mutation corresponds to a position (Asp4) in the XRCC1 structure that is thought to form a salt bridge at the BRCT dimer interface (36). Although M1652 would not be expected to be involved in salt bridge formation at neutral pH, residues in this region nevertheless may also be involved in homo- or heterodimer formation in BRCA1.

Missense mutations at positions 1702, 1703, and 1705 of the BRCT-N domain and a pentapeptide insertion at position 1822 of the BRCT-C domain abolish transcription activation by the BRCA1 COOH terminus (Tables 1 and 2). These mutations are predicted to occur in a region of highly variable length and composition that encompasses helix α2 in BRCT domains (36). It was suggested that this variability indicated that this region was not involved in formation of the core fold of the BRCT domain (36). Nevertheless, the mutations isolated here reveal that this region of the BRCT domain is critical for the transcription activation function of the BRCA1 COOH terminus.

Residue F6 forms part of a highly conserved hydrophobic pocket centered on residue W74 in helix α3 in the COOH-terminal BRCT domain of XRCC1 (36). Mutations at the corresponding position (F1761) in the BRCT-C domain of BRCA1 abolish transcription activation (Table 1). By analogy with XRCC1, residue F1761 of BRCA1 is also predicted to form part of a hydrophobic pocket, the disruption of which by mutation may compromise correct BRCT domain folding. In contrast, residue L25 is implicated in the interactions between helices α1 and α3, which form a paired helical bundle in the three-dimensional structure of the BRCT domain of XRCC1 (36). A missense mutation of the corresponding residue (L1780) or a pentapeptide insertion at this position in the BRCT-C domain of BRCA1 abolishes transcription activation by the BRCA1 COOH terminus region (Tables 1 and 2). These mutations are likely to affect the interactions between helices α1 and α3, thereby destabilizing the BRCT domain structure. Two other missense mutations in the BRCT-C domain, P1806A and V1833E, were shown, respectively, to display wild-type activity and to abolish transcription activation (Tables 1 and 3). Interestingly, P1806A is predicted to have no obvious effect on the structure, whereas a less drastic mutation at position V1833 (to methionine) has been predicted to destabilize the fold of the domain (36), suggesting that V1833E will behave similarly.

Pentapeptide insertions in many of the predicted secondary structure elements in the COOH-terminal region of BRCA1 abolish transcription activation (Table 1 and Fig. 3). Some of these insertions are likely to disrupt formation of the correct BRCT domain core fold, *e.g.,* insertions in strand β2 (1676RGTPL) and in helices α2 (1822GVPLH) and α3 (1717WGTPF). In contrast, the 1780GVPQL insertion in helix α1 is predicted to be at the BRCT dimer interface and thereby may affect the association of this domain with another protein, *e.g.,* RNA helicase A, which interacts with BRCA1 through residues in helix α1 (17).

Different Roles of BRCT-N and BRCT-C. Our insertion mutagenesis results suggest that BRCT-C can tolerate insertions better than BRCT-N without affecting transcription activation function. In addition, BRCT-N is more highly conserved in other species than is BRCT-C (39, 40), suggesting a higher constraint for function. The BRCT-N seems to be very important for binding to RNA helicase A (17), although it seems to lack an independent activation domain (mutant Y1769X is inactive). The borders of BRCT-C coincide well with the limits of the MTD, but only in combination with BRCT-N are high levels of activation achieved (13). It is tempting to speculate that BRCT-N is involved in the interaction of BRCA1 with RNA helicase A and is responsible for presenting BRCT-C in a correct way to obtain a transcriptionally competent activator.

Functional Assay. We have performed an extensive analysis of the BRCA1 COOH terminal region (amino acids 1560–1863) and have found that there is a correlation between loss of transcription activation function and the human genetic data, suggesting that the assay could be used to predict the effect of missense mutations in this region. Although the effects of mutations on transcriptional activity have been found to be comparable in yeast and mammalian cells (13, 15), it is possible that the effect of some mutations may be evident only in mammalian cells, *e.g.,* because of an interaction with mammalian-specific regulators, raising the possibility of a misinterpretation of the data obtained in yeast.

In the results presented here for substitution mutations, we have used a reporter gene with relatively low stringency (eight Lex operators; Ref. 26). The rationale for this choice was to recover only mutants that cause dramatic reduction or complete loss of activity. Mutations that partially disrupt the function would still activate the reporter. In the absence of knowledge of the minimum *in vivo* threshold of transcription activity needed for tumor suppression, it would be inappropriate to make decisions on whether a particular mutation would represent a wild-type or a cancer predisposing allele. For example, a particular mutation that shows 50% loss of activity in yeast could still be perfectly functional in breast and ovarian cells.

In conclusion, the data presented here suggest that the yeast assay for monitoring transcription activation by BRCA1 will provide a wealth of functional information in a research setting. That includes identifying protein-protein interaction regions, defining critical residues for activity, and providing tools to identify possible regulators. A general use of the assay to help in risk assessment and providing information for clinical decisions must await further confirmation from population-based studies.

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